Intellectual Property Office of New Zealand
Te Pou Rāhui Hanga Hou

CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 25 September 1998 with an application for Letters Patent number 332085 made by GLYCOX CORPORATION LIMITED.

I further certify that John Richard Baker is believed to be the true and first inventor of this invention.

Dated 26 June 2003.

Neville Harris
Commissioner of Patents

332085

NEW ZEALAND

PATENTS ACT, 1953

PROVISIONAL SPECIFICATION

"Fructosamine Oxidase Assay:
Methods and Materials"

We, GLYCOX CORPORATION LIMITED, a company duly incorporated under the laws of New Zealand of the offices of Bowden Impey & Sage, 470 Parnell Road, Auckland 1, New Zealand, do hereby declare this invention to be described in the following statement:

The Current Invention

The present invention relates to methods and materials for the assay of fructosamine oxidase enzyme in patients and particularly but not solely those predisposed to or with diabetes mellitus.

Diabetes mellitus is a common disease characterised by serious long-term vascular complications. Diabetic individuals have a 25-fold increase in the risk of blindness, a 20-fold increase in te risk of renal failure, a 20-fold increase in the risk of amputation as a result of gangrene, and a 2- to 6-fold increased risk of coronary artery disease and ischaemic brain damage. See, Klein R, Klein B, Davis M, DeMets D. *Diabetes Care* 8:311-5 (1985). Almost half those diagnosed as diabetic before the age of 31 years, die before they reach 50 years largely as a result of cardiovascular or renal complications, often with many years of crippling and debilitating disease beforehand. See, Deckert T, Poulsen J, Larsen M. *Diabetologia* 14:363-70 (1978).

Elevated blood glucose levels are now regarded as ***causative*** of diabetic complications based on results of the Diabetes Complications and Control Trial (DCCT). See, The Diabetes Control and Complications Trial Research Group. *N Eng J Med.* 379:977-85 (1993). The DCCT demonstrated that the development of microvascular complications of diabetes are related with degree of hyperglycaemia and that long-term complications may be ameliorated by rigorous treatment. After controlling for current HbA_{1c} levels, the development of microvascular complications in DCCT patients was strongly correlated with the degree of nonenzymatic glycation of structural proteins such of skin collagen, but not with advanced glycation end product (AGE) markers such as pentosidine, carboxymethylysine, and tissue fluorescence (V Monnier - personal communication). These findings imply that the nonenzymatic glycation of tissue proteins has greater pathophysiological importance than AGE formation.

Many of the features of diabetic vascular disease may also be attributed to oxidative stress, defined as an increase in the steady-state level of reactive oxygen or oxygen radicals in a biological system See, Baynes JW. *Diabetes* 40;405-12 (1991). For example, superoxide anions increase intracellular calcium which modulates the activity of nitric oxide synthase in the endothelium. Nitric oxide is a potent vasodilator and it has been implicated in the vascular dysfunction of early diabetes See, Ido Y, Kilo C, Williamson JR. *Nephrol Dial Transplant* 11 Suppl 5:72-5 (1996). Reactive oxygen species precipitate a drastic dose-dependent decrease in *de novo* synthesis of heparan sulphate proteoglycans leading to a reduction in anionic sites on the basement membrane and to an increased permeability to positively charged proteins such as albumin See, Kashira N, Watanabe Y, Makin H, Wallner EI, & Kanwar YS. *Proc Natl Acad Sci USA* 89:6309-13

(1992). Such leaky capillaries manifest clinically as background retinopathy and microalbuminuria. Microalbuminuria, in turn, is a recognised risk factor both for diabetic nephropathy in IDDM and for coronary artery disease and sudden death in elderly NIDDM See, Mogensen CE, Christensen CK. *N Eng J Med* 311;89-93 (1984) & Mogensen CE, Damsgaard EM, Froland A, et al *Clin Nephrol* 38 (suppl 1);s28-39 (1992).

Once natural anti-oxidant defences are exceeded, there is the potential for hydroxyl radical generation from superoxide via a copper catalysed Haber-Weiss reaction See, Halliwell B & Gutteridge JMC "Free radicals in Biology and Medicine" Clarendon Press, Oxford (pp. 136-76 1989). Hydroxyl radicals are extremely reactive species that cause serious site-specific damage. Oxygen radicals have also been implicated in the oxidative modification of low density lipoprotein (LDL) See, Witztum JL. *Br Heart J* 69; S12-S18 (1993). Oxidised LDL is a specific risk factor for atherosclerosis, binding with a scavenger receptor on tissue macrophages leading to the formation of foam cells and to cholesteryl ester accumulation in the intimal fatty streak, a feature of atheromatous plaque formation.

To date, the source of the oxidative stress in diabetes has not been identified. I have recently isolated an extracellular enzyme from the blood plasma of patients with diabetes mellitus which catalyses the elimination of fructosamines from glycated proteins. The reaction is potentially important because fructosamine is the precursor of all the Maillard products. Based on its high specificity for glycated protein substrates and its use of oxygen as acceptor, the enzyme may be classified as **fructosamine oxidase 1.5.3** See, Enzyme nomenclature, Recommendations of the Nomenclature Committee of the International Union of Biochemistry, Academic Press, London pp. 19-22, (1979). Fructosamine oxidase is a metalloenzyme with copper and quinone cofactors. Reaction products are free unglycated protein, α-dicarbonyl sugar, and superoxide (Figure 1).

Fructosamine oxidase belongs to the copper amine oxidase family of enzymes isolated from bacteria, fungi, yeast, and mammalian sera which are responsible for the oxidative deamination of biogenic amines See, Janes SM, Mu D, Wemmer D et al. *Science* 248;981-87 (1990). Structurally, fructosamine is a secondary amine (FIGURE 1) and it should be a substrate for such enzymes. A recent study has demonstrated that copper amine oxidase activity determined with benzylamine substrate is elevated in the plasma of individuals with diabetes mellitus compared with non-diabetic controls, and particularly in those people with microvascular diabetic complications. See, Boomsma F, Derkx FH, van den Meiracker AH, Veld AJ, Schalekamp MA. *Clin Sci Colch,* 88;675-9,(1995). I contend that the authors have not recognised the true nature of the enzyme measured, that the copper amine oxidase activity measured in the above study is, in fact, fructosamine oxidase, and that fructosamine oxidase activity is responsible for both the

oxidative stress and the long-term complications of diabetes. Furthermore, by employing a benzylamine substrate, the method of assay used by Boomsma et is not specific for glycated protein (fructosamine), it is relatively insensitive and labour-intensive, and it requires special analytical equipment that is not widely available. See, Van Dijk J, Boomsma F, Alberts G et al. *J Chromatogr 663;43-50 (1995).*

Summary of the Invention

The present invention relates to methods of monitoring fructosamine oxidase inhibition and/or antagonism of patients, screening patients to determine patients at risk to vascular (particularly microvascular) damage and identifying those individuals who will benefit by treatment with fructosamine oxidase in hibitors and/or antagonists, methods of determining fructosamine oxidase levels in a mammal, methods of determining blood plasma fructosamine oxidase levels in a diabetic individual or a suspected individual, methods of assaying blood serum or blood plasma *in vitro* for fructosamine oxidase and to related methods and procedures.

Accordingly, in one aspect the present invention consists in **a method of screening mammalian patients** (preferably humans suffering from or predisposed to diabetes) to determine patients at risk to vascular (particularly microvascular) damage, which method comprises determining the levels of fructosamine oxidase and/or the superoxide reaction product (or any other oxygen free radical product) of fructosamine oxidase in the population of patients and making the determination dependant upon such levels.

Preferably said screening is of blood taken from each patient.

Preferably the measurement conducted *in vitro* is of the superoxide reaction product (or any other oxygen free radical product) of fructosamine oxidase.

Preferably at risk patients are then treated **inter alia** to inhibit and/or to antagonise the fructosamine oxidase.

Preferably the procedure is substantially as hereinafter described.

In still a further aspect, the present invention consists in **a method of identifying those individuals who will benefit by treatment with fructosamine oxidase inhibitors and/or antagonists,** which method comprises testing an individual or a group of individuals for fructosamine oxidase in their blood directly or by reference to the superoxide reaction product (or any other oxygen free radical product) of fructosamine oxidase.

Preferably at risk patients are then treated **inter alia** to inhibit and/or to antagonise the fructosamine oxidase.

Preferably the procedure is substantially as hereinafter described.

In still a further aspect, the present invention consists in a **method of monitoring**

fructosamine oxidase inhibition and/or antagonism of a patient which comprises or includes testing (directly or indirectly) the fructosamine oxidase level of such patient.

Preferably such testing is by reference to the superoxide reaction product (or any other oxygen free radical product of fructosamine oxidase) in the blood of the patient.

Preferably each of the methods involves a determination of a particular level attributed to fructosamine oxidase and/or the reaction products referred to in comparison to such level or levels of a patient or patients (as the case may be) who is or are not at risk to such vascular damage, or will not benefit by treatment with fructosamine oxidase inhibitors and/or antagonists or have no need for fructosamine oxidase inhibition and/or antagonism.

In still a further aspect the present invention consists in **the measurement *in vitro* of the superoxide reaction product (and/or any other oxygen free radical product) of fructosamine oxidase** in the blood of a mammal by exploiting its reductant properties or its oxidant properties or by enzymatic means.

In one preferred form said measurement procedure involves (preferably at a pH 7 to 8 (most preferably at pH greater than 7.5)) the disabling of the superoxide scavenging mechanism (such as superoxide dismutase) (SOD) [e.g. using potassium cyanide] and then exposure [e.g. by addition] to a suitable fructosamine oxidase substrate [e.g. glycated bovine serum albumin modified to eliminate copper chelating activity which might disable the fructosamine oxidase].

Preferably the measurement following from the preferred procedure described involves a consideration [e.g. measurement] of an absorbance change, chemiluminescent change, or some other characterising change in an indicator of the modified sample.

In still a further aspect the present invention consists in a **method of determining the fructosamine oxidase levels in a mammal** (human or non-human) which at least includes procedures as previously set forth.

In still a further aspect the present invention consists in a **method of determining blood plasma fructosamine oxidase levels in a diabetic individual or a suspected diabetic individual** which comprises at least steps of a method as previously set forth.

In still a further aspect the present invention consists in a **method of assaying blood serum or blood plasma *in vitro (directly and/or indirectly)* for fructosamine oxidase** which involves at least one or more of the steps or procedures hereinbefore described and/or hereinafter described.

In still a further aspect the present invention consists in a **blood serum or blood plasma sample** of a patient in which the superoxide scavenging mechanisms therein have been disabled and the pH is in the range from 7 to 8.

Preferably said sample also includes or has been modified by exposure to a suitable

fructosamine oxidase substrate.

Preferably said fructosamine oxidase substrate is glycated bovine serum albumin modified to eliminate copper chelating activity which might disable fructosamine oxidase.

In still a further aspect the present invention consists in **the use of a sample** in accordance with the present invention for the purpose of any of the methods previously set forth.

The attention of the reader is drawn to my simultaneously filed New Zealand patent application in which there are disclosed a variety of procedures, methods, pharmaceutical compositions, dosage units etc. involving the use of fructosamine oxidase inhibition and/or antagonism in order to reduce vascular (preferably microvascular) damage to patients (particularly although not solely diabetic or suspected diabetic patients).

Preferably any such inhibitor or antagonist is selected from the groups

(i) copper chelating agents (eg: ethylenediamine tetraacetic acid, *o*-phenanthroline and histidine)

(ii) substrate analogues (eg: *N*-acetylcysteine, Nα-CBZ-L-arginine, captropril and enalapril).

(iii) hydrazine compounds (eg: diaminoguanidine, hydralazine and carbidopa).

The full content of the simultaneously filed patent specification is hereby included by way of cross reference.

Brief Description of the Drawings

Figure 1 shows a detailed reaction mechanism for the formation of fructosamine and Maillard products from glucose and protein. Fructosamine oxidase degrades fructosamine by a two-step reaction with initial release of an α-dicarbonyl sugar and subsequent oxidation of the enzyme/protein complex to release free unglycated protein. The reduced copper cofactor is oxidised *in vivo* by molecular oxygen and the oxidation product is superoxide.

Figure 2 shows the relationship between *fructosamine oxidase* measurements and plasma fructosamine. Linear regression equation ($y = 0.0349x - 5.9589$; $r^2 = 0.7455$).

Detailed Description of the Invention

(i) Assay principle

Fructosamine oxidase catalyses the degradation of fructosamine(s) with concurrent reduction of molecular oxygen yielding a superoxide reaction product (FIGURE 1). Superoxide is unstable in aqueous solution with spontaneous dismutation to hydrogen peroxide and oxygen. The dismutation reaction is strongly pH dependent with maximal reactivity in acidic solutions and reducing reactivity in alkaline solution. Therefore, enzyme activity is best determined at pH values 7.0-8.0 and preferably about pH 7.5 where superoxide is more stable using one of the assay compounds listed in Table 1.

TABLE 1

Assay compound	Assay pH	Type of reaction	Reference
Ferricytochrome c	7.8	Reduction	McCord J & Fridovich I. *J Biol Chem* 244;6087-93 (1969)
Nitroblue tetrazolium	7.8	Reduction	Halliwell B *FEBS Lett* 72;8 (1976)
Dichlorphenol indophenol	7.0	Reduction	Greenstock CL & Ruddock GW. *Int J Radiat Phys Chem* 8;367 (1976)
Epinephrine	7.8	Oxidation	Misra HP & Fridovich I *J Biol Chem* 247;3170-5 (1972)
Hydroxylamine	7.8	Oxidation	Elstner EF, Heupel A. *Anal Biochem* 70;616-20 (1976)
Peroxidase	7.8	Enzymatic	Misra HP, Fridovich I *Anal Biochem* 79;553-60 (1977)
NADH...LDH	7.0	Enzymatic	Chan PC & Bielski BHJ. *J Biol Chem* 249;1317-9 (1974)
NADH...GDH	7.2	Enzymatic	Chan PC & Bielski BHJ. *J Biol Chem* 255;874-6 (1980)

(ii) Interference

Because superoxide is potentially a noxious substance, superoxide degrading enzyme, superoxide dismutase (SOD), is elaborated in plasma as a physiological response

to increasing superoxide concentrations. Compared with healthy non-diabetic individuals, SOD levels are significantly elevated in the plasma of patients with diabetes mellitus and particularly amongst those patients with microvascular disease such as diabetic nephropathy and diabetic retinopathy. See, Mizobuchi N, Nakata H, Horimi T, Takahashi I. *Rinsho Byori* 41;673-8 (1993). The major SOD isoenzyme in extracellular fluids like plasma is extracellular SOD which is a tetrameric glycoprotein that contains four copper atoms and four zinc atoms. See, Karlsson K & Marklund SL *Biochem J* 242;55-9 (1987). Unless it is disabled, such SOD activity will cause significant interference in any blood plasma assay based on the detection systems listed in Table 1. Almost all of the SOD activity of human plasma is sensitive to inhibition with millimolar concentrations of potassium cyanide. See, Marklund SL, Holme E, Hellner L *Clin Chim Acta* 126;41-51 (1982). Alternative methods of disabling plasma SOD include adding low concentrations of sodium azide and sodium fluoride.

(iii) Procedure

Fructosamine oxidase activity may be measured using the redox-active colour reagent, ferricytochrome c, which is readily reduced by superoxide to form ferrocytochrome c with a characteristic increase in absorbance at 550nM (ϵ_{550} = 22.1 $mM^{-1}.cm^{-1}$). The reagent is 50mM TES buffer pH 7.4 containing 10μM cytochrome c (Sigma), 1000μM KCN, and 50μM fructosamine as glycated bovine serum albumin. The parameters for performance of the assay in a Cobas Bio (Roche) automated analyser are as shown in Table 2.

TABLE 2

	PARAMETER LISTING	
1	UNITS	U/L
2	CALCULATION FACTOR	473.9
3	STANDARD 1 CONCENTRATION	0
4	STANDARD 2 CONCENTRATION	0
5	STANDARD 3 CONCENTRATION	0
6	LIMIT	0
7	TEMPERATURE [DEG.C]	30.0
8	TYPE OF ANALYSIS	6
9	WAVELENGTH [NM]	550
10	SAMPLE VOLUME [UL]	5
11	DILUENT VOLUME [UL]	45
12	REAGENT VOLUME [UL]	200
13	INCUBATION TIME [SEC]	300
14	START REAGENT VOLUME [UL]	25

15	TIME OF FIRST READING [SEC]	0.5
16	TIME INTERVAL [SEC]	300
17	NUMBER OF READINGS	2
18	BLANKING MODE	1
19	PRINTOUT MODE	1

One unit of enzyme was defined as the amount which reduced 1 µmol/minute of cytochrome c in solution under the above assay conditions. The calculation factor is determined from the molar absorptivity for ferrocytochrome c (ϵ_{550nm}) according to the formula:

$$U/L\ (\mu mol.min^{-1}.L^{-1}) = TV \times 10^3 / \epsilon_{550} \times SV$$

where TV = total reaction volume
SV = sample volume

(iv) Materials

Glycated bovine serum albumin substrate was prepared as follows:

(a) Bovine serum albumin (BSA) (Sigma) was reduced with sodium borohydride to eliminate protein hydroperoxides. BSA (60g/L) was dissolved in 0.145M NaCl, pH was adjusted to 9.0 with molar NaOH, sodium borohydride (200mmol/L) was added, and the solution was stirred gently at room temperature for 24 hours. Excess sodium borohydride was discharged with glacial acetic acid and the solution was dialysed exhaustively against 0.145M NaCl at 4 °C.

(b) Borohydride-reduced BSA was glycated by mixing protein solution with an equal volume 0.4M Na_2PO_4 buffer pH 7.4 containing 50mM glucose and 0.02% sodium azide and incubating at 37 °C for 7 days. Excess glucose was removed by exhaustive dialysis against 0.145M NaCl.

(c) Glycated BSA (gBSA) was acetylated by adding 0.2M iodoacetic acid, adjusting pH to 6.8, and incubating at room temperature for 24 hours. Excess iodoacetate was removed by exhaustive dialysis against 0.145M NaCl.

(d) Remaining copper binding sites on gBSA were saturated by dialysing against 0.145M NaCl containing 100µM copper sulphate. Excess copper was removed by exhaustive dialysis against 0.145M NaCl.

(e) Degree of glycation of gBSA substrate was determined by fructosamine assay (Hoffmann La-Roche).

(v) Substrate specificity

The specificity of the assay for reactive oxygen species was tested by measuring the degree of inhibition of ferricytochrome c reduction after adding the following oxygen free radical scavengers to the reaction mixture: (a) Superoxide dismutase to selectively remove superoxide; (b) catalase to selectively remove hydrogen peroxide; & (iii) mannitol to scavenge hydroxyl radicals. Results are shown in Table 3.

TABLE 3

Free radical scavenger	Enzyme activity* (U/L)	Significance (P)
Control	15.34 ± 0.16	-
superoxide dismutase (20kU/L)	9.99 ± 0.03	<0.0001
catalase (1000kU/L)	12.23 ± 0.03	<0.0001
superoxide dismutase + catalase	6.78 ± 0.12	<0.0001
mannitol (50mmol/L)	14.96 ± 0.19	0.0421

- determined with free radical scavenger added to the reagent.

Results imply that the assay reaction is measuring both superoxide and hydroxyl radicals formed from the reaction of superoxide with hydrogen peroxide.

(v) Specificity

Cytochrome c is a non-specific reductant and other reducing substances in sera or anticoagulants added to the blood sample at specimen collection may interfer in the assay as shown in Table 4.

TABLE 4

Additive*	Activity compared with control (%)
Control	100
Heparin (1000U/L)	24.4
EDTA (100μM)	26.3

- Human *fructosamine oxidase* analysed with and without (control) additive in the reagent

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(vi) Comparison with fructosamine concentrations

Fructosamine oxidase activity was measured in non-diabetic sera and results were compared with serum fructosamine concentrations Figure 2.

DATED THIS 25th DAY OF September 1998
A.J. PARK & SON
PER Tabatha O'Leary
AGENTS FOR THE APPLICANT

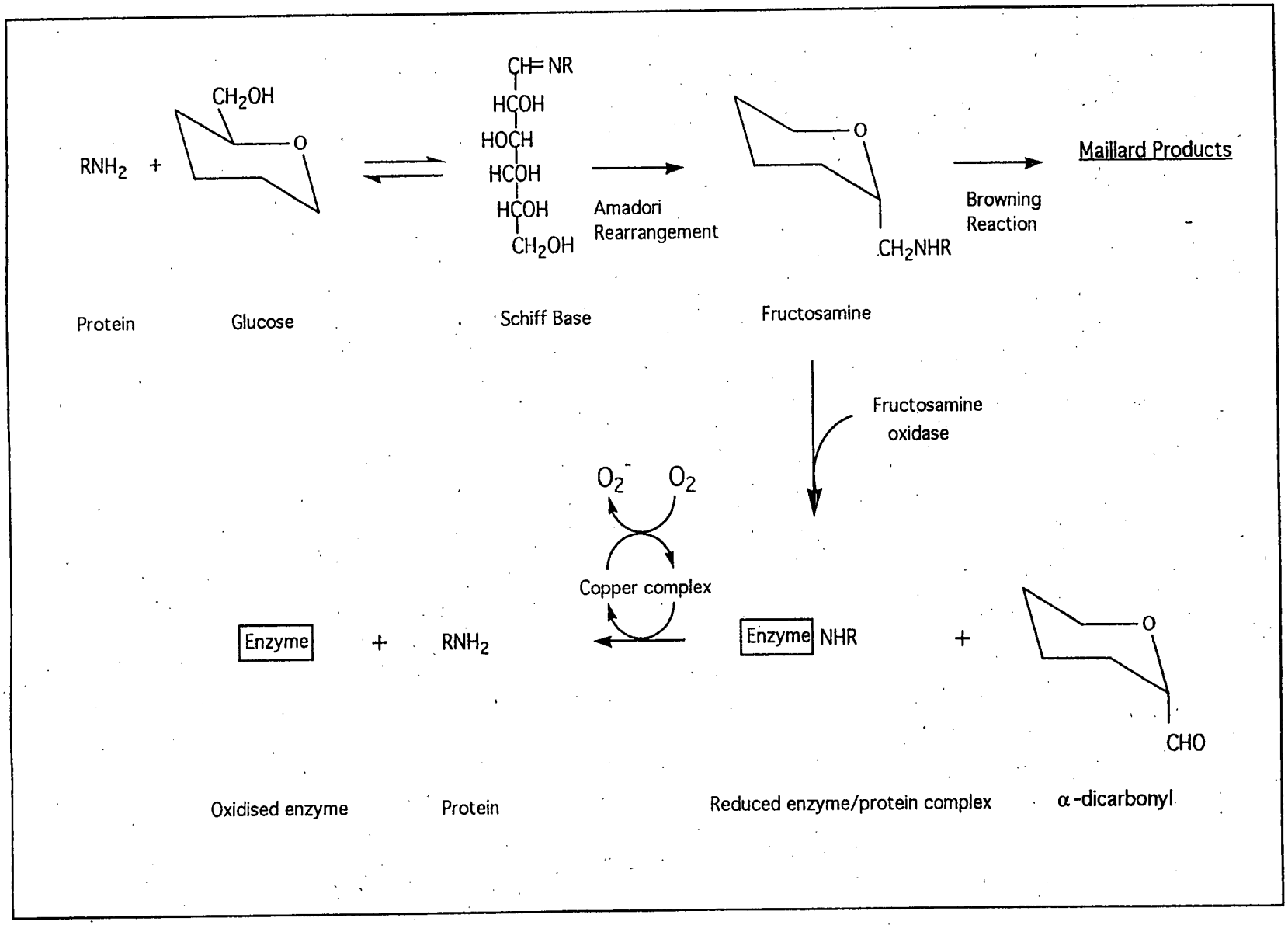
RNH2 +
CH2OH
O
CH=NR
HCOH
HOCH
HCOH
HCOH
CH2OH
Amadori Rearrangement
O
CH2NHR
Browning Reaction
Maillard Products
Protein
Glucose
Schiff Base
Fructosamine
Fructosamine oxidase
O2- O2
Copper complex
Enzyme
+
RNH2
Enzyme NHR
+
O
CHO
Oxidised enzyme
Protein
Reduced enzyme/protein complex
α-dicarbonyl

FIGURE 1.